Original Research Paper

Effects of intrathecal injection of rapamycin on pain threshold and spinal cord glial activation in rats with neuropathic pain

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Objective: To evaluate the effects of intrathecal injection of rapamycin on pain threshold and spinal cord glial activation in rats with neuropathic pain.

Methods: Healthy 30 male Sprague Dawley (SD) rats were randomly divided into six groups (n=5 in each group): (1) control group without any treatments; (2) chronic constriction injury (CCI) group; (3) Early-rapamycin group with intrathecal injection of rapamycin 4 hours after CCI days; (4) Early-vehicle group with intrathecal injection of dimethyl sulphoxide (DMSO); (5) Late-rapamycin group with intrathecal injection of rapamycin 7 days after CCI; (6) Late-vehicle group with intrathecal injection of DMSO 7 days after CCI. Rapamycin or DMSO was injected for 3 consecutive days. Mechanical and thermal threshold were tested before and after the CCI operation. Lumbar segment of spinal cords was tested for glial fibrillary acidic protein (GFAP) by immuohistochemistry on 14th day after operation.

Results: Mechanical and thermal hyperalgesia emerged on fourth day were maintained till fourteenth day after operation. After intrathecal injection of rapamycin 4 hours or 7 days after CCI, mechanical and thermal threshold significantly increased compared to injection of DMSO. The area of GFAP positive and the mean density of GFAP positive area in the dorsal horn of the ipsilateral side greatly increased in rapamycin-treated groups.

Conclusions: Intrathecal injection of rapamycin may attenuate CCI-induced hyperalgesia and inhibit the activation of astrocyte.

Keywords: Rapamycin, Neuropathic pain, Intrathecal injection, Astrocyte

Introduction

Neuropathic pain has pain hypersensitivity properties, such as spontaneous pain, hyperalgesia and allodynia.[1] Previous evidence showed that this hypersensitivity might be associated with neuronal plasticity including both peripheral and spinal neuronal circuits.[2] Neuropathic pain caused by injury or disease is associated with peripheral or central nerve system. The original injury is usually healed without visible tissue damage. Shortly after the injury, the pain is paroxysmal with abnormal sensation, spontaneous pain and relentless pain caused by normally non-painful stimuli (allodynia) or increased long-term sensitivity to pain caused by mild painful stimuli (hyperalgesia).[3] The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that widespread throughout the nervous system. Recent study showed that mTOR is closely related with pain sensation and participates in regulating synaptic plasticity change.[4] Two distinct mTOR complexes, mTORC1 and mTORC2, were found in the cells. The difference was characterised by different components and their functions.[5]

It is reported that mTORC1 regulated the protein translation process by phosphorylating the downstream proteins, such as eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and p70 ribosomal S6 protein kinase (p70S6K).[6] Recently, a direct role of mTOR in the modulation of glial functions has also been identified and previous studies revealed that mTOR is involved in microglial proinflammatory activation.[7] Rapamycin is an mTORC1 inhibitor that can block mTORC1 activity and downstream targets.[8] Recent studies showed that the intrathecal administration of rapamycin could alleviate the symptoms of neuropathic pain including

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the increased mechanical hypersensitivity.[9,10] In this study, we have focussed on the effect of intrathecal injection of rapamycin on the pain-related behaviour and activation of astrocyte in the spinal cord dorsal horn of chronic constriction injury (CCI) rat model. Our investigation on the function of mTOR during the development and maintenance of neuropathic pain might provide theoretical basis for discovering new target in neuropathic pain treatment.

Materials and Methods

Experimental animals and drugs

This work was approved by the Ethic Committee of Guangzhou First People's Hospital. Adult male Sprague Dawley (SD) rat weighing 220–260 g, provided by the Laboratory Animal Centre of Guangdong province, were maintained in a light-controlled room (lights on from 7:00 a.m. to 7:00 p.m.) at a constant temperature of 26 ± 2°C.

Chronic constriction injury

Chronic constriction of the sciatic nerve was performed according to the method described by Bennett.[11] Briefly, rats were anaesthetised with 10% chloral hydrate (300–350 mg/kg, i.p.) for surgical procedures. The left sciatic nerve was exposed at the level of the mid-thigh. Four ligatures (4–0 chromic gut, Ethicon, Rome, Italy) were tied loosely around the sciatic nerve with a 1.0 mm interval between each ligature. There should be a slight vibration in the calf muscles without affecting the blood supply in the outer membrane. The wound was then closed in layers.

Rapamycin solution

Rapamycin (R5000-50MG) was purchased from LC Laboratories (USA) and was dissolved in 4% DMSO diluted in saline. The working concentration was 1 μg/ml and stored at 4°C.

Intrathecal catheterisation and drug delivery

Micro spinal catheter was implanted in the lumbosacral cord according to modified Yaksh's method.[12] Rats were anaesthetised with 10% chloral hydrate (300–350 mg/kg, i.p.) and were laid on the surgical board in prone position. An incision was made through skin and fascia above the basilar clivus. Blunt separation of muscles was performed to expose the atlanto-occipital membrane and pierce with syringe needle so that cerebral solution gushed from the hole and vibrated with the breath. Implant the polyurethane micro spinal catheter into lumbar slowly through the wound. The catheter was 7.5–8 cm in length and was fixed by connecting with a 1.5 cm length polyethylene. The wound was closed in layers after the surgery. Intramuscular injection of penicillin on the right upper limb was performed to prevent infection. Paralysis of both lower limbs within 30 seconds occurred after injection intrathecally through the catheter with 20 μl of 2% lidocaine, and then disappeared after 30 minutes, indicating a successful intrathecal catheterisation. Those with sensory and motor disturbance, such as monoplegia, paraplegia or hemiplegia, were excluded 2 days after the catheterisation.

Intrathecal injection of rapamycin (10 μg/10 μl) 4 hours after CCI was performed for consecutive 3 days (once a day) in the early-rapamycin group. The same operation was performed on the early-vehicle group with injection of 10 μl 4% DMSO instead of rapamycin. Intrathecal injection of rapamycin 10 μg/10 μl or 10 μl 4% DMSO were performed in the late-rapamycin group and late-vehicle group, respectively, every day from seventh to ninth day after CCI. Rats without any treatments were served as control group.

Sample collection and processing

Rats were sacrificed by cervical dislocation under deep anaesthesia with 10% chloral hydrate through i.p. injection (300–350 mg/kg) in the fourteenth day after CCI. Lumbar spinal cord segments L4–L5 were collected on ice and a small piece of the left afferent nerve was kept to separate the left and right side. Part of the collected samples was frozen in liquid nitrogen for protein and RNA extraction, and the left was fixed in 4% phosphate-buffered paraformaldehyde overnight, and then kept for dehydration with gradient ethanol, incubated in 95, 70, 50 and 30% ethanol for 2 minutes, each sample was then sliced into 3 μm thickness by RM135 microtome.

Thermal and mechanical pain threshold determination

Thermal pain threshold was determined by using 37370 radiant thermal stimulator. The rats were placed on a thin glass platform. The radiant light focussed on the pelma directly. The infrared (I.R.) Intensity was 60% and the stimulating time was 20 seconds to avoid tissue damage. The paw withdrawal thermal latencies (PWTL) were recorded automatically. The mean latency was determined by five independent tests excluding the maximum and minimum.

Mechanical pain threshold was assessed by the paw withdrawal mechanical threshold (PWMT) in response to mechanical stimuli by using 2390 von Frey. Single rat was placed in a transparent plastic cage with a wire mesh bottom. The filaments were applied to the plantar surface of the hind paw. The smallest filament eliciting paw withdrawal was considered as the threshold. Five trials with 5-minute intervals were applied for each animal. The mean threshold was determined by five trials except the maximum and minimum.

Semi-quantitative analysis of GFAP

Motic professional series optical microscope was used to obtain the images of glial fibrillary acidic protein (GFAP) expression after immunohistochemistry. The GFAP positive areas and average densities were assessed by Motic Image Advanced 3.0. The mean positive areas and average densities were determined by three different high quantitative (400 ×) regions in one slide.

Statistical analysis

Data was presented as mean ± standard deviation (SD). SPSS software was used to perform statistical analysis. One-way ANOVA was use for comparison between different groups. Pairwise comparison was conducted by LSD and SNK-*q* test. Whereas, Student's *t*-test was used for comparisons within groups. *P* value less than 0.05 indicates significant difference.

Results

The changes of animals' pain thresholds

We tested the thermal withdrawal latency and mechanical withdrawal threshold as indicators of pain thresholds every other day after the CCI surgery and 1 day before CCI. We found that the thermal and mechanical withdrawal thresholds of CCI group (Tables 1 and 2) were decreased from the fourth day similar with those of the control groups. However, thermal and mechanical withdrawal thresholds of early-rapamycin group were significantly increased comparing with those in CCI group from day 4 to day 14 ($P<0.05$) and thermal and mechanical withdrawal thresholds of late-rapamycin group had significant difference comparing with those in CCI group since day 8 ($P<0.05$). These results indicated that mechanical and thermal hyperalgesia emerged on fourth day and maintained till fourteenth day after CCI operation and intrathecal injection of rapamycin 4 hours or 7 days after CCI could increase the mechanical and thermal thresholds.

GFAP expressions in the spinal dorsal horn

We analysed the fluorescence densities of GFAP after immunohistochemistry. We just injured the left side sciatic nerve, thus the right side was regarded as control side. We found the GFAP positive areas and the average densities were similar in the control side ($P>0.05$) (Table 3). The GFAP positive areas and the average densities of injury side were significantly increased in CCI group, early-rapamycin groups and late-rapamycin groups comparing with those in control group, although the areas and densities were not significantly different among CCI group, NS-early and NS-late groups ($P>0.05$). However, the GFAP-positive areas and the average densities in Rapamycin treatment groups with intrathecal injection of rapamycin 4 hours or 7 days after CCI were significantly decreased than those in CCI group ($P<0.05$). Moreover, we found GFAP-positive area in rats of late-rapamycin group was smaller than that in early-rapamycin group ($P<0.05$), while the average densities was similar in both groups ($P>0.05$).

Table 1 Thermal withdrawal latency of all groups

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Control	7.96 ± 0.98	8.02 ± 0.84	8.04 ± 0.89	7.95 ± 0.89	8.13 ± 1.17	7.96 ± 0.77	7.93 ± 0.81	8.08 ± 0.86
Chronic constriction injury(CCI)	8.06 ± 0.97	7.35 ± 0.84	4.92 ± 0.66*	4.57 ± 0.91*	4.06 ± 0.59*	4.16 ± 0.73*	4.29 ± 0.71*	4.21 ± 0.76*
ER	7.85 ± 0.87	7.58 ± 0.86	6.73 ± 0.88	6.85 ± 0.59	6.66 ± 0.94	5.97 ± 0.60*	6.51 ± 0.73*	6.15 ± 0.79*
EV	7.90 ± 0.68	6.67 ± 1.00*	5.13 ± 0.79*	4.54 ± 0.83*	3.88 ± 0.67*	4.23 ± 0.54*	4.09 ± 0.93*	4.50 ± 1.15*
LR	7.94 ± 1.18	6.88 ± 1.04	5.25 ± 0.91*	4.15 ± 1.06*	5.39 ± 1.19*	5.82 ± 0.91*	5.31 ± 0.60*	5.41 ± 0.90*
LV	8.22 ± 1.04	6.84 ± 0.64	5.27 ± 0.81*	4.83 ± 0.52*	4.37 ± 0.84*	3.81 ± 0.79*	4.21 ± 0.95*	4.38 ± 0.85*

Data was shown as mean ± standard deviation (SD).ER: early-rapamycin group; EV: early-vehicle group; LR: late-rapamycin group; LV: late-vehicle group. * $P<0.05$ comparing with control; $P<0.05$ comparing with CCI.

Table 2 Mechanical withdrawal threshold of all groups

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Control	39.93 ± 4.20	41.12 ± 3.68	38.84 ± 2.34	41.15 ± 3.76	41.13 ± 3.49	41.39 ± 3.05	39.95 ± 2.81	41.87 ± 2.22
Chronic constriction injury(CCI)	39.51 ± 2.97	36.17 ± 2.04	19.98 ± 1.97*	19.93 ± 1.91*	17.42 ± 1.39*	18.86 ± 2.07*	20.06 ± 2.62*	2.87 ± 3.36*
ER	39.92 ± 2.53	37.13 ± 2.00	37.23 ± 2.10	34.43 ± 3.71	28.09 ± 1.65	27.34 ± 3.57	25.21 ± 3.49	26.45 ± 3.53
EV	39.97 ± 2.15	35.59 ± 3.36*	30.04 ± 2.70*	20.42 ± 2.28*	18.62 ± 2.01*	18.36 ± 1.72*	18.85 ± 1.66*	21.16 ± 3.44*
LR	40.37 ± 2.96	36.53 ± 2.19	29.86 ± 3.40*	19.41 ± 2.76*	26.40 ± 3.13*	29.23 ± 3.48*	28.99 ± 2.98*	31.21 ± 2.49*
LV	40.80 ± 3.04	35.49 ± 3.45	30.33 ± 2.54	21.13 ± 2.17*	19.87 ± 2.91*	18.20 ± 1.57*	17.65 ± 2.16*	19.26 ± 2.26*

Data were shown as mean ± standard deviation (SD).ER: early rapamycin group; EV: early vehicle group; LR: late rapamycin group; LV: late vehicle group. * $P<0.05$ comparing with day 0; $P<0.05$ comparing with CCI.

Table 3 Glial fibrillary acidic protein (GFAP) positive areas and densities (mean ± SD)

	Control	Chronic constriction injury (CCI)	ER	EV	LR	LV
Positive areas(L)	57 836 ± 5644.4	113 128 ± 8286.5*	95 407 ± 8247.5*	108 061 ± 7995.9*	84 984 ± 2459.5*	109 226 ± 11 119.9*
Positive areas(R)	60 271 ± 5746.5	61 286 ± 3609.9	60 623 ± 4027.2	60 669 ± 7775.1	60 805 ± 5167.3	60 854 ± 8656.8
Densities (L)	0.2230 ± 0.0014	0.2405 ± 0.0019*	0.2269 ± 0.0011*	0.2387 ± 0.0016*	0.2276 ± 0.0021*	0.2375 ± 0.0012*
Densities (R)	0.2219 ± 0.0049	0.2294 ± 0.0095	0.2217 ± 0.0089	0.2349 ± 0.0097	0.2206 ± 0.0113	0.2204 ± 0.0039

Data were shown as mean ± standard deviation (SD).L: left; R: right; ER: early-rapamycin group; EV: early-vehicle group; LR: late-rapamycin group; LV: late-vehicle group. * $P<0.05$ comparing with day 0; $P<0.05$ comparing with CCI.

Discussion

The present study showed that intrathecal injection of rapamycin could alleviate increased mechanical and thermal thresholds and activities of astrocytes after CCI in rat models. Our study indicated the function of mTOR during the development and maintenance of neuropathic pain, this might provide theoretical basis leading to new target in neuropathic pain treatment.

Neuropathic pain may be caused by trauma or disease that affects the peripheral or central nerve system. The symptoms included spontaneous pain, hyperalgesia and allodynia. Widely used animal models for studying the mechanisms of neuropathic pain are CCI, partial sciatic nerve ligation (PSNL) and spinal nerve ligation (SNL).[13] chronic constriction injury model was first reported by Bennette *et al.* in 1988, this model had mimicked multiple features of clinic neuropathic pain.[11] In this study, we found after CCI surgery, the thermal withdrawal latency and mechanical withdrawal threshold of injure side were significantly decreased than those in control side (Tables 1 and 2), and rats had lifting and licking foot behaviour, these indicated that we have successfully established the neuropathic pain model.

Mammalian target of rapamycin is a member of phosphatidylinositol 3-kinases (PI3K) family. Recent studies showed that mTOR regulated cell proliferation, neuronal development and synaptic plasticity.[4] When activated by neurotrophic factor or hormone, mTOR could have anti-apoptosis effect through regulating the protein translation processing and phosphorylated the downstream proteins.[14] In this study, we intrathecally administrated rapamycin, an mTORC1 inhibitor within 4 hours after CCI surgery. Then, we monitored rats' pain thresholds, we found that in days 4–14 after CCI surgery, rats receiving rapamycin treatment had higher pain threshold than those of normal saline administration. These indicated that intrathecal administration of rapamycin could decrease rats' pain hypersensitivity after CCI. Moreover, pharmacological intervention before or during chronic neuropathic pain also had the similar effect.

Glial cells can support neuronal function through providing necessary nutrition. Moreover, glial cells have important roles in formation and development of neuropathic pain.[15] Evidence showed that glial cells in spinal cord could be activated during peripheral nerve injury or cancerous pain.[16,17] In this study, we found the activities of glia in rat's spinal dorsal horn have significantly increased. The immunohistochemistry signal of GFAP was increased, mainly in the first and second levels of spinal dorsal horn. We observed that the cell bodies of astrocytes were enlarged, the processes were thicker and longer and the number was increased. These increased astrocytes' activities were correlated with the decreased pain threshold after CCI surgery. However, intrathecal administration of rapamycin could increase the mechanical withdrawal threshold and thermal withdrawal latency. Simultaneously, the GFAP area and average density were significantly decreased. It indicated that the activities of astrocytes were decreased. These results suggested that rapamycin might decrease the pain hypersensitivity through inhibiting astrocytes' activities.

In conclusion, our study suggested that mTOR signalling had important role in the development of neuropathic pain and intrathecal administration of rapamycin could alleviate the symptom of neuropathic pain. Thus, mTOR signalling might be therapeutic target for neuropathic pain.

Disclaimer Statements

Contributors Guarantor of integrity of the entire study: Shouzhang She; study concepts and study design: Shouzhang She and Jing Lv; definition of intellectual content: Jing Lv; literature research: Jing Lv; clinical studies: Jing Lv, Zhenci Li and Yanlu Ying; experimental studies: Jing Lv, Zhenci Li and Yanlu Ying; data acquisition: Jing Lv, Zhenci Li and Yanlu Ying; data analysis: Jing Lv and Lixin Xu; statistical analysis: Jing Lv and Lixin Xu; manuscript preparation, editing and review: Jing Lv and Shouzhang She.

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Conflicts of interest The authors have no conflict of interest to disclose.

Ethics approval This work was approved by the Ethic Committee of Guangzhou First People's Hospital.

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